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SCIENTIFIC OPINION

Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition[1†]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others. In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g. mutation, deletion) must be demonstrated. For other species, concerns appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in human has not yet been established. In the absence of animal models shown to be able to distinguish hazardous from non hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. If the strain proves to be cytotoxic it is not recommended for use.

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KEY WORDS

Bacillus species, enterotoxin production, emetic toxin, cereulide, surfactin-like lipopeptides

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[1] On request from EFSA, Question No EFSA-Q-2013-00303, adopted on 8 April 2014.

[†] This guidance document replaces the previous EFSA Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition, adopted in November 2011 (EFSA-Q-2009-00973). The requirements for the assessment of species belonging to the *Bacillus cereus* group are unchanged.

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) to update the FEEDAP Panel Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition published in 2011.

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others.

In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g. mutation, deletion) must be demonstrated.

For other species, concerns appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in human has not yet been established. In the absence of animal models shown to be able to distinguish hazardous from non hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. If the strain proves to be cytotoxic it is not recommended for use.

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BACKGROUND AS PROVIDED BY EFSA

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Article 7(6) of this Regulation provides for the European Food Safety Authority (EFSA) to publish detailed guidance to assist applicants in the preparation and presentations of applications.

EFSA has the responsibility to assess the safety of feed additives before an authorisation is granted. A considerable amount of feed additives are composed by microorganisms. As a tool to simplify and harmonise within EFSA the assessment of microorganisms used in food and feed, the Scientific Committee published in 2007 one opinion on the introduction of a Qualified Presumption of Safety (QPS) approach for the assessment of selected microorganisms.

The list of microorganisms included in such opinion and considered to qualify for the QPS approach to safety assessment is updated regularly by the Biological Hazards (BIOHAZ) Panel. The last update is from 2012. The QPS approach is regularly used by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in the assessment of microbial products subject to a pre-authorisation assessment.

Bacillus species are widely used as feed additives, and several of them are considered to qualify for the QPS approach to safety assessment, provided that the qualification of the absence of food poisoning toxins, surfactant activity or enterotoxic activity is met. In 2000, the Scientific Committee for Animal Nutrition (SCAN) adopted an opinion on the safety of use of *Bacillus* species in animal nutrition. This opinion was revised in 2011 by the FEEDAP Panel in the form of the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2011), and updated according to the most recent scientific and technical developments. The aim of this document, which complements the QPS opinion, is to provide applicants with proportionate and up-to-date guidance on how to conduct the safety assessment of *Bacillus*-based products.

This Guidance makes a clear difference between the *Bacillus cereus* group (including known human enteropathogens) and other *Bacillus* species.

Science evolves fast and since the Guidance document was issued, new information on the toxicity and prevalence of these toxins has become available. Therefore, the FEEDAP Panel in view of this and of the experience gained so far from the assessment of the toxigenic potential of products based on *Bacillus* species (other than *B. cereus*) is intended to produce an update of the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. This output is aimed at highlighting the uncertainties and making proposals to address them in the context of the assessment of the dossiers of non-*Bacillus cereus* based products.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The FEEDAP Panel is requested to update the Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition.

1. Introduction

A number of strains of *Bacillus* species are used in animal production either directly as microbial feed additives or as the source of other feed additives, notably enzymes. Regulation (EC) No 1831/2003 requires that all feed additives, including microorganisms, are assessed for safety before being placed on the market. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* (and related genera) is a capacity for toxin production. However, the capacity for toxin production is unevenly distributed over the genus, occurring frequently in some species and more rarely, if at all, in others. For this reason, the Scientific Committee on Animal Nutrition (SCAN), when first developing guidance in this area, recommended that the use of strains of the *Bacillus cereus* taxonomic group, a group containing many known pathogenic strains, be strongly discouraged. However, the Committee recognised that strains from other *Bacillus* species may be considered safe (EC, 2000). The FEEDAP Panel concurs with this general position.

The Qualified Presumption of Safety (QPS) approach to the safety assessment of microorganisms adopted by EFSA is considered applicable to most of the commercially relevant *Bacillus* species (EFSA, 2007; EFSA BIOHAZ Panel, 2013). This approach requires the unambiguous identification of the strain being assessed, a demonstration of susceptibility to clinically relevant antibiotics and, in particular, evidence that the strain lacks a capacity for toxin production. Any other strain of *Bacillus* or related genera not falling within the scope of the QPS approach would also require an assessment of toxigenic potential. This document is intended to provide technical guidance for the assessment of any toxigenic potential for strains of *Bacillus* intended to be used directly as a feed additive or indirectly as a source of such additives.

2. The scope of the guidance

Although a number of species earlier considered to belong to the genus *Bacillus* have been transferred to other genera, to date none has been the subject of a feed additive assessment. Since relatively little is known about the toxigenic capacity of the genera related to *Bacillus* (i.e., *Geobacillus, Aneurinibacillus* and *Paenibacillus*) and, consequently, whether the approach to safety assessment described would fully apply, it is considered prudent to restrict this guidance to bacterial strains belonging to *Bacillus sensu strictu.*

3. Safety concerns caused by *Bacillus* species

3.1. Identification

Characterisation of *Bacillus* strains according to Claus and Berkeley (1986) and Bergey’s manual of Systematic Bacteriology (2009) must be completed by molecular methods to identify strains to the species level. This is essential as it determines whether the current guideline applies and, if so, the nature of the testing recommended. Partial sequences (approximately 500 bp) of the 16S rRNA gene can be amplified using methods described in Guinebretière et al. (2001) and From *et al*. (2005) and compared to sequences from databases. If the partial sequence does not provide a definitive identification, then the 16S rRNA gene should be fully sequenced (Guinebretière *et al*., 2001). To differentiate species within the *B. subtilis* group, partial sequences of the *gyrA* gene or *gyrB* genes may be needed in addition to the 16S rRNA gene sequences. These can be obtained using methods described in Chun and Bae (2000) and From et al. (2005) for *gyrA* and Wang et al. (2007) for *gyrB*.

3.2. Assessment of *Bacillus* species other than the *Bacillus cereus* group

Bacillus species other than members of the *B. cereus* group are a rare cause of foodborne diseases. In such events, the food contained high numbers (between 10^5 and 10^9 CFU/g) of the suspected *Bacillus* spp. (Kramer and Gilbert, 1989, From et al., 2007a). The production of the *B. cereus*-like diarrhoeal enterotoxins by some strains of other *Bacillus* species was described in the SCAN opinion (EC, 2000), although such strains have so far not been associated with foodborne diseases. The current view is that the very few reports of *B. cereus*–like enterotoxins occurring in other species of *Bacillus* are likely to have resulted from a misidentification of the strain involved (From et al.*,* 2005). The few incidents of

food poisoning investigated where non-*B. cereus* group strains were determined to be the causative organism suggest an association with surfactin-like lipopeptides (From et al., 2007b). However, the capacity for cyclic lipopeptides production appears widely distributed, if not universal amongst strains of *B. subtilis* (Apetroaie-Constantin et al., 2009, From *et al*., 2007a, Hwang et al., 2009, Mikkola et al., 2007), *B. licheniformis* (Nieminen et al., 2007, Dybwad et al., 2013, Madslien et al., 2013), *B. pumilus* (Taylor et al., 2005, From et al., 2007b) and *B. mojavensis* (From et al., 2005). The relation between the presence of surfactin-like lipopeptides and/or other toxic factors and the risk of illness in human has not yet been established.

In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products.

Accordingly, the following is recommended for the assessment of non-*B. cereus* group species:

A cytotoxicity test made preferably with Vero cells or other epithelial cell lines using culture supernatant following the protocol described in the Appendix. Detection based on ^{14}C-leucine uptake is described but the use of other methods such as those based on lactate dehydrogenase release or propidium iodide uptake could be used as an alternative (Fagerlund et al., 2008).

If the strain proves to be cytotoxic it is not recommended for use.

The FEEDAP Panel recognises that the underlying cause of the rare outbreaks of food poisoning attributed to non-*B. cereus* species is currently poorly understood. Accordingly, the Panel would be open to alternative approaches to establish the safety of these *Bacillus* strains.

3.3. Assessment of species belonging to the *Bacillus cereus* group

A review of the virulence factors involved in the gastro-intestinal infections caused by *B. cereus* can be found in Stenfors-Arnesen et al. (2008):

- The role of hemolysin BL (Hbl) and of the non-hemolytic enterotoxin (Nhe) in diarrhoeal outbreaks has been confirmed (Stenfors-Arnesen et al., 2008). In particular the mode of action of Nhe on the cell membranes has been described (Lindbäck et al., 2010). Genes coding for Nhe, unlike those coding for Hbl, are present in most, if not all, strains of *B. cereus* (Guinebretière et al., 2010, Fagerlund et al., 2007) and the amount of Nhe produced at 32 °C by *B. cereus* strains was correlated with their cytotoxic activities (Moravek et al., 2006).
- The toxin previously named 'Enterotoxin K' (now cytotoxin K) has been characterised as a beta-barrel cytotoxin now called CytK (Lund et al., 2000). Two forms are distinguished (Fagerlund et al., 2004), CytK1 being more cytotoxic than CytK2.
- Enterotoxin T has now been identified as the result of a cloning artefact (Hansen et al., 2003) and should no longer be considered as a virulence factor.
- Enterotoxin FM has been identified as an endopeptidase (Tran et al., 2010) which does not show direct toxic activity on epithelial cells.
- Emetic toxin (cereulide) is still the only toxin identified in *B. cereus* causing the emetic disease. Its potent toxic effect on liver cells and various mammalian cell lines has been shown (Andersson et al., 2007). Fatal or very severe *B. cereus* emetic outbreaks have been reported since 2000 (Shiota et al., 2010; Posfay-Barbe et al., 2008; Dierick et al., 2005). The non-ribosomal peptide synthase producing cereulide has been identified (Ehling-Schulz et al., 2005) and characterised (Magarvey et al., 2006).

Other factors produced by *B. cereus* with various toxic activities have been characterised (Hemolysin II and several metalloproteases) but there is no evidence so far of their implication in gastro-intestinal

diseases (Cadot et al., 2010). The toxic effect some of them show on macrophages may rather indicate a role in clinical infections.

In summary, diarrhoeal disorders produced by *B. cereus* result from the production of toxins Nhe, Hbl and CytK, alone or in combination in the intestine (Table 1). The emetic disease results from the production of cereulide by *B. cereus* cells in the food.

Table 1: *Bacillus cereus* toxins which can be considered as the causative agents of gastro-intestinal diseases (Stenfors-Arnesen et al., 2008)

Toxin	Genes/operons	Nature	Foodborne infection/intoxication
Nhe (non hemolytic enterotoxin)	*nhe*	Protein (three components)	diarrhoeal
Hbl (hemolysin BL)	*hbl*	Protein (three components*)	diarrhoeal
CytK (cytotoxin K)	*cytK*	Protein	diarrhoeal
Cereulide	*ces*	Cyclic peptide	emetic

* The production of a fourth component, whose role has not been elucidated, was shown by Clair et al., 2010.

In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable.

If, however, they are proposed for use then the full genome (including chromosome and plasmids) should be sequenced and bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase (Table 1). If there is evidence of homology, the non-functionality of the genes (e.g., mutation, deletion) should be demonstrated.

Strains harbouring a toxigenic potential should not be used as feed additives.

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APPENDIX

Recommended procedure for the detection of cytotoxicity using epithelial cell lines

1. Preparation of test substance

Bacterial cells should be grown in brain heart infusion broth (BHI) at 30 °C and harvested after 6 h when it is anticipated that cells will have reached a density of at least 10^8 CFU/mL. Cells should be removed by centrifugation at room temperature. Toxicity is determined using 100 µL of supernatant in the Vero cells assay.

2. Cell assay

Vero cells are grown in MEM medium supplemented with 5 % foetal calf serum. Cells are seeded into 24-well plates two-three days before testing. Before use, check that the growth of the Vero cells is confluent. If so, remove the medium and wash the cells once with 1 mL preheated (37 ºC) MEM medium.

- Add 1 mL preheated (37 ºC) low-leucine medium to each well and then add the test substance (1-100 µL of *Bacillus* supernatant), incubate the cells for 2 hours at 37 ºC.

- Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37 ºC) low-leucine medium. Mix 8 mL preheated low-leucine medium with 16 µL ^{14}C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 hour at 37 ºC.

- Remove the radioactive medium and add 1 mL 5 % trichloroacetic acid (TCA) to each well, incubate at room temperature for 10 minutes. Remove TCA, and wash the wells twice with 1 mL of 5 % TCA.

- After removing TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 minutes. Transfer the content of each well to liquid scintillation tubes with 2 mL of liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 minute.

- Percentage inhibition of protein synthesis is calculated using the following formula: ((Neg. ctrl − sample)/Neg. ctrl) × 100; the negative control is Vero cells from wells without addition of sample. Above 20 % inhibition is considered to indicate cytotoxicity.

For the alternative method with propidium iodide uptake or lactate dehydrogenase, values above 20 % of the fluorescence/absorbance obtained from the positive control (usually detergent treated cells) are considered to indicate cytotoxicity.

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